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Contributions towards a specific DHA enrichment in the live food *Brachionus plicatilis* and *Artemia* sp.

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ABSTRACT: This paper reports on enrichment techniques oriented towards a specific DHA accumulation in the live prey *Brachionus plicatilis* and *Artemia sp.* The product used in these experiments (DHA7) is an experimental emulsion with a DHA/EPA ratio 6.7:1. Rotifers enriched with this pure DHA7 emulsion contained up to 68 mg/g DW DHA and a DHA/EPA ratio of 1.6. The DHA/EPA ratio could be increased to a much higher level by different enrichment techniques. Dilution of the DHA7 emulsion with coconut oil prior to enrichment also had a beneficial effect on the DHA/EPA ratio of the rotifers. Using a mixture DHA7/coconut oil 3:7 the DHA/EPA ratio could be raised to 4.9. In *Artemia* DHA levels were slightly influenced by changing the salinity during the enrichment procedure. However, scrutinized research oriented towards DHA accumulating *Artemia* strains may be more appropriate.

1 INTRODUCTION

Supplementation of (n-3) essential fatty acids to finfish larvae through the live prey *Brachionus plicatilis* and *Artemia sp.* has been well documented in literature. The incorporation of (n-3) HUFA in the live prey is obtained by different enrichment procedures with different unicellular algae (Watanabe et al. (1979)), oil emulsions (Watanabe et al. (1983a,b); Léger et al. (1986); Bengtson et al. (1991)), microcapsules (Walford and Lam (1987)) or commercial enrichment diets (Artemia Systems, Baasrode, Belgium).

Using these techniques, considerable improvements have been achieved in the nutritional quality of the live prey and the exact (n-3) requirements of some species could be established. However, big controversion still exists on which polyunsaturated lipids constitute an "optimal" or even "desirable" dietary balance, and in which proportion (n-3),(n-6) and (n-9) fatty acid families should be incorporated in the diet (Sargent et al. (1991). The requirements for (n-3) lipids can indeed be met by different (n-3) neutral or polar lipids with different effectiveness.

It has been observed that for marine fish the developing eggs and larvae preferentially consume docosahexaenoic acid (DHA) over eicosapentaenoic acid (EPA) as a more efficient energy or prostaglandin source (Watanabe (1978)). Supplementation of DHA in the live diets during the early feeding stage of red seabream, yellowtail, striped jack, striped knifejaw and flounder improved the growth, survival, stress resistance and prevention of hydrops (Watanabe (1993)). In addition, it is believed that the ratio DHA over EPA may be an important factor in the pigmentation of some flatfish (Devresse et al. (1992)) and that unbalanced ratios in live prey could finally lead to fatty acid ratios in the body of the fish which largely diverge from the values which are found from wild caught animals.

All these observations and findings have created a sudden demand in enrichment products with high DHA concentration and high DHA/EPA ratio. These enrichment products are, however, all extracted from fish or animal tissue and thus quite difficult to procure. For extra DHA enrichment the aquaculturist is thus still restraint to the use of microalgal species belonging to the brown algal class *Prymnesiophyceae* (*Isochrysis sp.*) or commercial products, such as, DHA SUPER SELCO recently launched by Artemia Systems, SA (Belgium).

In this paper special attention will be paid to the kinetics of DHA in the rotifer *Brachionus plicatilis* and the brine shrimp *Artemia sp.* Several enrichment techniques will be discussed in which

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the final concentration of DHA or the DHA/EPA ratio will be modified by a simple change in physico-chemical parameters or the use of specific *Artemia* strains.

2 MATERIAL AND METHODS

Rotifers were reared on an algal replacement diet (Culture Selco, CS; Artemia Systems) according the method described in Lavens et al. (1993). All enrichments by oil emulsion were performed at a density of 200 rotifers per ml, using two separate dosages of 150 ppm at 3 hours interval. The salinity and temperature of the water were maintained at 25 ppt and 27 °C respectively. Newly-hatched *Artemia* were also concentrated to a density of 200 individuals per ml and enriched during 24 h using the oil emulsion at a concentration of 0.6 g/l. Temperature and salinity during enrichment were kept at 27°C and 35 ppt respectively.

Fatty acid analyses were performed following the standard ICES procedure (Artemia Reference Center, 1993): sample extraction was performed with a $CHCl_3/CH_3OH$ (2:1) solvent mixture; non-lipid contaminants were removed with a KCl solution and dehydrated over anhydrous Na_2SO4. Transesterification of lipids was obtained by using CH_3COCl/CH_3OH (1h/100°C). After adding internal standard (20:2(n-6)-methylester), the fatty acid methyl esters (FAME's) were dissolved using hexane, and the solvents were evaporated. The FAME's were diluted in iso-octane and injected on column in a Carlo Erba Mega Series 5160 gaschromatograph with the following characteristics: FID, column 25 m BPX-70 (very polar), 0.32 mm internal diameter. Peak identification and quantification were done with a Spectrophysics Integrator SP4270. The results of all fatty acid analysis are presented as mg FAME g^{-1} dry weight.

CULTURE SELCO was obtained from Artemia Systems. The oils (DHA7 and DHA20) were obtained by different extraction methods and used as emulsions or diets. The EPA and DHA concentrations obtained after fatty acid analysis of the experimental emulsions and diets are given in Table I. The characteristics of the oils which were used to dilute DHA7 are represented in Table II.

3 INCORPORATION OF DHA IN ROTIFERS

Although rotifers synthesize DHA *de novo*, the rate of synthesis is rather low and largely insufficient for supplying the essential fatty acid in sufficient amounts to fast-growing fish larvae (Lubzens et al. (1985)). Hence, rotifers need to be boosted (bioencapsulated) with DHA prior to feeding to fish larvae.

In our experiments, bioencapsulation of DHA was obtained by two different enrichment methods (Table III). In the indirect enrichment method rotifers were first reared on CULTURE SELCO (CS) using the method described by Lavens et al. (1993). Rotifers obtained by this method contained more EPA than DHA which resulted in a DHA/EPA ratio lower than 1. In order to lower down the EPA content of the rotifers the latter were exposed to a diet of baker's yeast (BY) for 3 consecutive days prior to enrichment in a separate tank.

Table I. EPA and DHA concentrations in the experimental emulsions and diets.

	EPA (mg/g DW)	DHA (mg/g DW)	DHA/EPA	Σ(n-3)HUFA ≥20:3(n-3)
Experimental emulsions				
DHA7	67.2	452.3	6.7	550.6
DHA20	0.8	15.6	19.5	16.4
Experimental diets				
CS (commercial)	18.9	15.3	0.8	36.4
DHA-CS	16.9	26.7	1.6	45.4
DHA-PS	24.4	70.6	2.9	99.3

Table II. Chemical characteristics of the oils used in the dilution of DHA7.

Fatty acid	Coconut oil	(n-6) oil	(n-9) oil
(n-3)	0.2	7.7	0
(n-6)	0.2	58.5	8.7
(n-9)	26.9	7.7	75.4
% saturates	63.3	24.0	13.9
% monoenes	28.5	0.3	76.3
% dienes	5.4	58.5	8.8
% trienes	0.4	7.4	0.9
% remainder*	0.4	0.4	0

*remainder=fatty acids containing 4, 5 and 6 double bounds

Table III. EPA, DHA and (n-3) HUFA concentrations in rotifers in function of different enrichment methods.

	EPA (mg/g DW)	DHA (mg/g DW)	DHA/EPA	Σ(n-3)HUFA ≥20:3(n-3)
Indirect method				
CS	6.6	4.8	0.7	13.9
CS + DHA7	41.4	68.0	1.6	116.8
CS + 3days BY	0.7	0.5	0.7	1.5
CS + 3days BY + DHA7	15.5	44.9	2.9	65.1
CS + 3days BY + DHA20	0.8	6.3	7.9	8.4
Direct method				
CS	6.6	4.8	0.7	13.9
DHA-CS	4.3	5.9	1.4	12.4
DHA-CS-PS	4.4	12.9	2.9	19.9
Isochrysis	2.3	10.7	4.7	22.7

In the direct enrichment method the food (CS) was replaced by an experimental DHA-CS during four consecutive days and followed by an experimental DHA-PROTEIN SELCO (PS) for 1 day. The advantage of this method resides in the fact that no extra enrichment is needed and that no extra space is required for a separate enrichment. As a comparison with the natural enrichment technique, rotifers reared on *Isochrysis* were also included in the study.

It is clear from Table III that the indirect enrichment method offers the possibility to reach high DHA and HUFA levels when the rotifers are immediately transferred to the oil emulsion. However, the initial DHA/EPA balance which is lower than 1 in rotifers reared on CS is not completely reversed after enrichment and results in a low DHA/EPA ratio which for some species might be too low. Offering the same emulsion to rotifers after they have depleted their DHA reserves is a possible solution to improve the DHA/EPA ratio but this is at the cost of the HUFA concentration. Using oils with high DHA/EPA proportions the DHA/EPA ratio of rotifers can even be raised above the levels which are generally obtained by algal enrichment with *Isochrysis*. The direct enrichment method has the advantage that the rotifers are reared on a full diet and do not need to be manipulated before enrichment. The rotifers obtained by this technique still have a lower DHA/EPA ratio than rotifers reared on *Isochrysis* but are considerably better than those fed on the commercial CS.

Table IV. EPA, DHA and (n-3) HUFA concentrations in rotifers in function of the length of the enrichment period.

Enrichment period (hours)	EPA (mg/g DW)	DHA (mg/g DW)	DHA/EPA	ΣHUFA (mg/g DW)
6	18.4	50.7	2.8	74.3
12	15.9	55.5	3.5	77.2
24	15.8	59.4	3.8	81.3

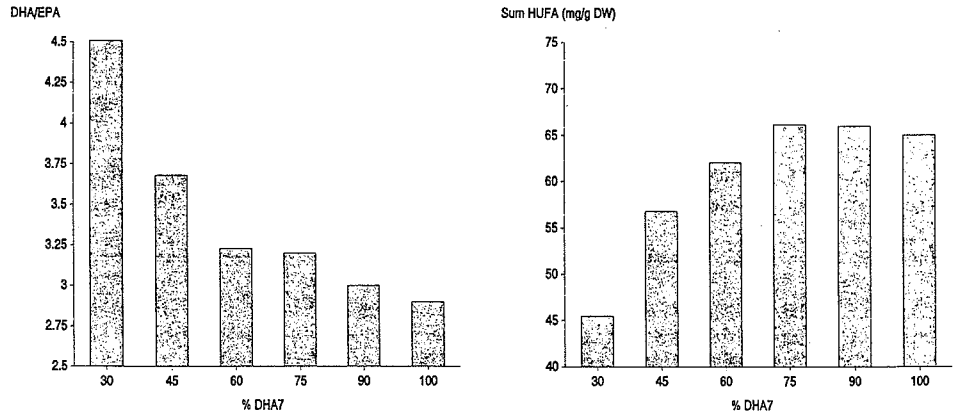

Figure 1: DHA/EPA ratio and HUFA content of rotifers enriched with a DHA7 emulsion diluted with coconut oil

In order to improve the DHA/EPA ratio other methodologies in the indirect enrichment method were tested. For this, rotifers were enriched with 300 ppm DHA7 and maintained in the emulsion for 6, 12 and 24 hours. The results of this experiment are given in Table IV.

It is obvious that prolonged immersion in the enrichment solution has a positive effect on the DHA accumulation while the EPA concentration in the rotifers is decreasing. Consequently, higher DHA/EPA ratio's are obtained after longer enrichment periods.

Another solution to obtain high DHA/EPA ratio's consists in diluting the DHA7 emulsion with other oils. Figure 1 illustrates the change in the DHA/EPA ratio in rotifers fed on a set of isolipidic emulsions where part of the DHA7 oil emulsion was substituted by progressively higher amounts of coconut oil. The data on the DHA/EPA ratio of the rotifers are inversely proportional to the amount of DHA7 in the emulsion which might indicate that coconut would play a protective role during catabolism. This hypothesis may be confirmed by the results of the total HUFA content in the rotifers where a plateau level is reached at a 75:25 DHA7/coconut ratio despite the higher HUFA content of the emulsion with 90 and 100% DHA7.

Other analogous enrichment experiments with the DHA7 emulsion were also undertaken but this time the dilution was obtained by using oils containing high levels of (n-6) and (n-9) fatty acids. In none of these experiments the DHA/EPA ratio of the rotifers was affected and the (n-6) and (n-9) fatty acids accumulated in the tissue of the rotifers according to their proportion in the emulsion.

4 INCORPORATION OF DHA IN *ARTEMIA SP.*

DHA retainment during and after enrichment is far more complicated in *Artemia* than in rotifers. This

can partly be explained by the different stage of the life cycle of both species. Most of the rotifers are indeed in the adult stage where metabolic activities are not as pronounced as in the larval *Artemia* stages. As a consequence the fatty acid composition of the rotifers is stable and does not change considerably during enrichment or starvation conditions. *Artemia* nauplii, on the other hand, do not synthesize DHA and metabolise the highly unsaturated fatty acids very fast. This could be observed in the experiments where isolipidic mixtures of DHA7 emulsion and coconut oil were administered to the nauplii. The DHA/EPA ratio stayed stable around 2 except for the highest dilutions (Figure 2).

If catabolism in GSL *Artemia franciscana* would be responsible for a fast DHA consumption, change in the environmental conditions during enrichment may be a way to prevent DHA reduction. In order to verify this hypothesis GSL *Artemia franciscana* were enriched with DHA7 at different salinities. The results of this exposure to different salinities are summarized in Table V.

It is clear that for lower salinities lower HUFA levels are obtained but the DHA/EPA ratio is not affected and has an average of 2.5 which is close to the maximum level which could be obtained in GSL *Artemia franciscana* with a DHA7 enrichment.

Also dilutions of the DHA7 emulsion with the (n-6) or (n-9) oils never resulted in an improved DHA/EPA ratio and resulted only in a proportional increase of the (n-6) or (n-9) fatty acids.

Since none of the manipulations with GSL *Artemia franciscana* resulted in an improved DHA/EPA ratio, the DHA7 enrichment was tried with a number of other *Artemia* strains (Table VI). The fatty acid profile of the newly hatched *Artemia* of the different strains was significantly different at the start. San Francisco Bay (SFB) and China 1242 *Artemia sp.* nauplii contained 10-15 times less 18:3(n-3) than the other strains. The levels of arachidonic acid were low in all strains except for SFB which contained 4.3 mg/g DW. The same strain also contained 4-5 more EPA than the rest. China 1242 nauplii were characterized by a very low (n-3) content which is compensated by a high (n-6) level and consequently results in a (n-6)/(n-3) ratio which is 10-15 times higher than in all the other strains. As a result the HUFA content in this strain is very low. SFB on the other hand had the highest HUFA content.

After enrichment with DHA7 the initial concentration of 18:3(n-3) and 20:4(n-6) hardly changed. All strains except SFB contained approximately 4 times more EPA after enrichment. SFB nauplii, who had the highest initial EPA content (16.1 mg/g DW), only ended up with an EPA content which was 1.5 times higher.
The Chinese strains (China 1197, China 1188) had a considerable accumulation of DHA which resulted in a very high DHA/EPA ratio and HUFA content.

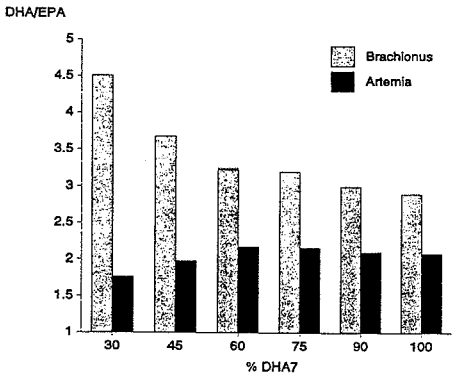

Figure 2: Comparison in the DHA/EPA ratio of rotifers and *Artemia franciscana* enriched with a DHA7 emulsion diluted with coconut oil

Table V. Changes in EPA, DHA and HUFA concentrations in GSL *Artemia franciscana* enriched with a DHA7 emulsion at different salinities.

Salinity (ppt)	EPA (mg/g DW)	DHA (mg/g DW)	Σ HUFA (mg/g DW)
5	10.7	27.5	43.5
35	13.1	31.5	48.9
55	16.9	40.3	62.4
75	17.8	45.7	69.3
95	16.9	42.1	64.2
115	16.9	39.4	61.3

5 CONCLUSIONS

In conclusion it can be said that next to the existing commercial products which aimed to copy the fatty acid profile of the green alga *Nannochloropsis* a second generation of products can be launched which approximate the lipid

Table VI. Fatty acid profile of various *Artemia* strains obtained on newly hatched nauplii (A) and 24 hours enriched nauplii on a 0.6 g/l DHA7 emulsion (B).

	NEWLY-HATCHED *ARTEMIA SP.* NAUPLII										
Strain collection number*	18:3(n-3)	20:4(n-6)	20:5(n-3) EPA	22:6 (n-3) DHA	DHA/EPA	(n-3)	(n-6)	(n-9)	(n-6)/(n-3)	(n-9)/(n-3)	ΣHUFA
GSL-S	48.6	0.7	3.4	0	0.00	54.2	11.2	23.7	0.21	0.44	6.0
SFB	2.4	4.3	16.1	0.6	0.04	20.3	13.3	22.8	0.66	1.12	17.9
PR China 1197	39.3	0.4	3.5	0.4	0.11	43.7	10.0	23.6	0.23	0.54	4.4
PR China 1188	25.8	1.6	4.1	0.4	0.10	30.8	12.9	17.2	0.42	0.56	5.0
PR China 1242	3.6	0.4	1.4	0.4	0.29	5.4	27.1	23.5	5.02	4.35	1.8
GSL-N	37.5	0.6	3.7	1.0	0.27	43.1	13.1	23.9	0.30	0.55	5.6

	ARTEMIA NAUPLII ENRICHED WITH DHA7 FOR 24 HRS										
Strain collection number*	18:3(n-3)	20:4(n-6)	20:5(n-3) EPA	22:6 (n-3) DHA	DHA/EPA	(n-3)	(n-6)	(n-9)	(n-6)/(n-3)	(n-9)/(n-3)	ΣHUFA
GSL-S	39.8	1.0	14.8	35.0	2.36	94.5	11.3	36.3	0.12	0.38	54.7
SFB	3.5	4.3	24.0	28.8	1.20	58.4	14.7	37.9	0.25	0.65	54.9
PR China #1197	32.6	0.7	14.0	51.4	3.67	101.9	12.0	42.4	0.12	0.42	69.3
PR China #1188	18.4	1.8	14.6	72.1	4.94	110.5	13.2	39.0	0.12	0.35	92.1
PR China #1242	4.2	0.7	8.8	22.8	2.59	37.5	23.1	30.8	0.62	0.82	33.3
GSL-N	37.0	1.0	12.6	34.9	2.77	88.1	14.3	45.3	0.16	0.51	51.1

* collection number (Artemia Reference Center - *Artemia* cyst bank)
GSL-S : Great Salt Lake (GSL) South Arm, Utah-USA (ARC #3442)
SFB : San Francisco Bay (SFB), California-USA (ARC #08 180 14)
GSL-N : Great Salt Lake (GSL) North Arm, Utah-USA (ARC #1248)

properties of *Isochrysis*.

The results obtained with the on rotifers suggest that depending on the choice of the enrichment procedure virtually any DHA concentration can be obtained together with a high DHA/EPA ratio. *Artemia sp.*, however, are far more difficult to manipulate and the final success in the enrichment is largely dependent on the genetic characteristics of the strain. In this respect, the excellent results obtained for some Chinese strains should encourage further research in order to evaluate their genetic and biological potential for aquaculture.

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